

Case Docket RAN No. 4039/42

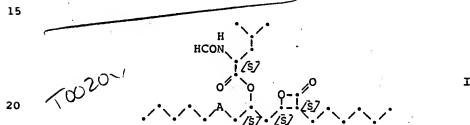
2-HEXYL-3-HYDROXY-⁵⁰¹ ~~LEUCINE DERIVATIVES~~ ~~HEXADECANOIC ACID LACTONE DERIVATIVES~~

Background

- 5 The present invention concerns N-formylleucine derivatives which are useful in the treatment of obesity and hyperlipaemia.

Summary of the Invention

10 The present invention is concerned with compounds of the formula

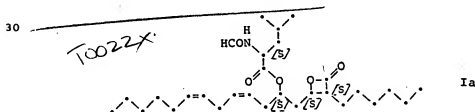


TOO21V B

wherein A signifies the group

or $(CH_2)_5$

25 Formula I above embraces (2S,3S,5S,7Z,10Z)-5-[(S)-2-formamido-4-methyl-valeryloxy]-2-hexyl-3-hydroxy-7,10-hexadecadienoic acid lactone of the formula



35 B (i.e., lipstatin), and (2S,3S,5S)-8-[(S)-2-formamido-4-methyl-valeranyloxy]-2-hexyl-3-hydroxy-hexadecanoic acid lactone of the formula

Nt/4.6.84

S1878 06/22/84 621827 - 2
S1879 06/22/84 621827

S1880 06/22/84 621827

08-2525 1 101
08-2525 1 102

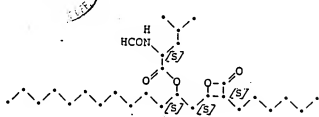
300.00CH
20.00CH

- 08-2525 1 103

30.00CH

ROOM
JUN 10
1964

100307



Ib

P-285

(i.e., tetrahydrolipstatin).

10

P. These compounds are novel and have valuable pharmacological properties. In particular, they inhibit pancreas lipase and can be used in the control or prevention of obesity and hyperlipaemia.

15

Objects of the present invention are the compounds of formula I above per se and as pharmaceutically active substances, the manufacture of these compounds, medicaments and industrially-produced foodstuffs containing a compound of formula I, their production as well as the use of these compounds in the control or prevention of illnesses.

116
21

25

30

35

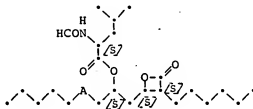
Detailed Description of the Invention

P

The present invention is concerned with compounds of the formula

10

10040V

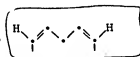


I

15

10041X

wherein A signifies the group
or $m(\text{CH}_2)_m$.



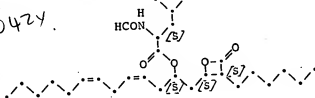
16

Formula I above embraces (2S,3S,5S,7Z,10Z)-5-[(S)-2-formamido-4-methyl-valeryloxy] — 2-hexyl-3-hydroxy-7,10-hexadecadienoic acid lactone of the formula

20

25

10042Y



Ia

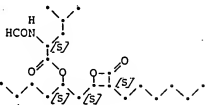
30

31

which is referred to hereinafter as lipstatin, and (2S,3S,5S)-5-[(S)-2-formamido-4-methyl-valeryloxy] — 2-hexyl-3-hydroxy-hexadecanoic acid lactone of the formula

35

T0050x



Ib

10 2 which is referred to hereinafter as tetrahydrolipstatin.

These compounds are novel and have valuable pharmacological properties. In particular, they inhibit pancreas lipase and can be used in the control or prevention of obesity and hyperlipaemia.

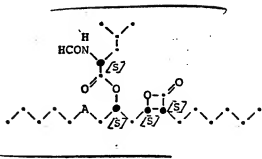
The present invention is also concerned with pharmaceutical compositions for preventing and treating obesity or hyperlipaemia comprising a compound of formula I and a pharmaceutically acceptable carrier material.

The invention also concerns methods for preventing or treating obesity or hyperlipaemia in an afflicted mammal wherein compound I is administered to the mammal in an amount which is effective in preventing or treating obesity or hyperlipaemia.

The invention also is directed to a commercially-produced foodstuff comprising compound I admixed with a material suitable for consumption.

819 As used herein, [S] indicates the absolute configuration of compound I in the S form. More particularly, the applicable carbon atoms in formula I having the S configuration are noted with enlarged circles in the following formula:

100607



10

wherein A is as above.

P The digestion of fats (triglycerides) taken in with the food is effected in the intestine by pancreas lipase. The pancreas lipase cleaves the primary ester bonds of triglycerides, whereby free fatty acids and 2-monoglycerides result as products. These products can then be resorbed and utilized. By inhibiting the pancreas lipase the aforementioned cleavage of the food fats and therewith also the resorption and utilization of these substances is partially prevented; the triglycerides are excreted in unchanged form.

The inhibition of pancreas lipase by the compounds of formula I can be demonstrated experimentally by registering titrimetrically the oleic acid liberated in the cleavage of triolein by pig pancreas lipase. An emulsion which contains 1 mM taurodeoxycholate, 9 mM taurodeoleate, 0.1 mM cholesterol, 1 mM egg lecithin, 15 mg/ml BSA (i.e., bovine serum albumine), 2 mM Tris-HCl,

100 mM sodium chloride, 1 mM calcium chloride and the substrate triolein is treated with the compound of formula I dissolved in ethanol or dimethyl sulphoxide (10% of the emulsion volume) and the reaction is started by the addition of 100 μ l (175 U) of pig pancreas lipase. The pH is held at 8 during the reaction by the addition of sodium hydroxide. The IC_{50} is calculated from the consumption of sodium hydroxide determined during 10 minutes. The IC_{50} is that concentration at which the lipase reactivity is inhibited to half of the maximum. Table I hereinafter contains IC_{50} values determined for the compounds of formula I and data concerning the acute toxicity (LD_{50} after single oral administration to mice).

Table I

Test compound	IC_{50} in μ g/ml	LD_{50} in mg/kg p.o.
Lipstatin	0.07	>4000
Tetrahydrolipstatin	0.18	-

The inhibition of the resorption of fats taken in with the food, which is brought about by the inhibition of pancreas lipase, can be demonstrated in a double-labelling experiment on mice. For this purpose, there is administered to the test animals a test meal, which contains 3H -triolein and ^{14}C -oleic acid, and a compound of formula I. By measuring the radioactivity there is then determined the amount of 3H -triolein and ^{14}C -oleic acid (in % of the amount

administered) excreted with the feces. The results set forth in Table II hereinafter show that in comparison to untreated control animals the excretion of unaltered tri-
 5 glyceride increases greatly and the excretion of oleic acid remains largely unchanged.

100804
 Table II

Test compound	Number of experimental animals	Dosage	Excretion in % of the amount administered			
			Triolein		Oleic acid	
Controls	12	-	3.5 [±]	0.3	10.1 [±]	0.6
Lipstatin	6	40 mg/kg *	56.8 [±]	13	13.8 [±]	5.6

* The experiments were carried out with a preparation which contained about 10% lipstatin. The dosage specified is the amount of lipstatin administered.

P The compounds of formula I can be manufactured in accordance with the invention by the following procedure:

25 P (a) for the manufacture of the compound of formula Ia, aerobically cultivating a microorganism of the species *Streptomyces toxytricini* which produces this compound in an aqueous culture medium which contains suitable carbon and
 30 nitrogen sources and inorganic salts and separating the compound of formula Ia produced from the culture broth, or

P (b) for the manufacture of the compound of formula Ib, hydrogenating the compound of formula Ia.

35 Streptomycetes strains which produce lipstatin, the compound of formula Ia, can be isolated from soil samples from various locations. An example is the microorganism

isolated from a soil sample found in Mallorca, Spain, which was given the laboratory designation Streptomyces sp. 85-13 and which has been identified by CBS, Baarn (Netherlands) as Streptomyces toxytricini Preobrazhenskaya & Sveshnikova (see Bergey's Manual of Determinative Bacteriology, 8th Edition, page 811). It thereupon received the new designation Streptomyces toxytricini 85-13. A lyophilized sample of this strain was deposited on the 14th June 1983 at the Agricultural Research Culture Collection, Peoria, Illinois, under the designation NRRL 15443.

A description of the identification of Streptomyces sp. 85-13 is given hereinafter:

Media

The composition of the media used is described in Int. J. Syst. Bacteriol 1966; 16, 3; 313-321.

Nonomura diagram

Nonomura used the results of the International Streptomyces Project (ISP) for the classification of the Streptomycetes species (J. Ferment. Technol. 1974, 52, 2).

Colours

The names and code numbers of the aerial mycelium come from Tresner & Backus "System of color wheels for streptomycete taxonomy". The colours of the reverse of the colonies come from H. Prauser's selection from Baumann's "Farbtonkarte Atlas I".

Methodology

This was carried out according to the ISP methods (see Int.

J. Syst. Bacteriol, 1966, 16, 3; 313-340).

u7. I. Agar cultures after 16 days at 28°C (double determination)

5 p (a) Oatmeal agar

Growth: abundant; colonies: thin, spreading; aerial mycelium: velutinous, pinkish brown (Light Brown 57); reverse of the colonies: yellowish (Pr. Co-3-m) with broad purple-grey (Pr. Oc-6-x) margin; soluble pigments: doubtful.

10 p (b) Starch-salt agar

Growth: good; colonies: thin, spreading; aerial mycelium: velutinous, pinkish brown (Light Brown 57) with white sectors; reverse of the colonies: dark straw coloured (Pr. Co-5-b) with some dark reddish brown [Pr. O-5-S(r)] spots; soluble pigments: doubtful. The diastatic action is excellent.

20 p (c) Glycerine-asparagine agar

Growth: good; colonies: thin, spreading; aerial mycelium: velutinous, pale pinkish brown (R4ec: Grayish Yellowish Pink); reverse of the colonies: orange (Pr. Oc-3-m/r); soluble pigments: pale pinkish brown.

25 a (d) Yeast malt agar

30 p Growth: good; colonies: thin, spreading; aerial mycelium: reddish brown (4ge: Light Grayish Reddish Brown 45); reverse of the colonies: yellow (Pr. Co-4-5) and dark brown (Pr. Oc-5-r); soluble pigments: very pale yellowish brown.

35 u20 II. Agar cultures after 62 days at 28°C (double determination)

u (a) Oatmeal agar

p Growth: good; colonies: thin, spreading; aerial mycelium:

powdery velutinous, cinnamon coloured [R-41e: Light Brown (57)-Cork Tan] with broad, paler margin [R. 5gc: Light Reddish Brown (4.2)-Peach Tan]; reverse of the colonies: yellowish-brown with ochre-yellow (Pr. Co-3-a) margin, slightly greyish towards the bright (Pr.Oc-4-r) centre; soluble pigments: pale ochre-brown.

(b) Starch-salt agar

As on oatmeal agar, but with a more greyish brown reverse (Pr. Oc-6-c) and with dark brown (Pr. Oc-4-r) spots and rings at the ends of the cross-hatches.

(c) Glycerine-asparagine agar

As on starch-salt agar, but paler light beige (Sec: Grayish Yellowish Pink 32-Dusty Peach); Reverse: ochre-yellow [Pr. Co (=Cr)-4-b], paler in the centre; no soluble pigments.

(d) Yeast-malt agar

Growth: fair; colonies: almost as on oatmeal agar, but with very thin, pale grey margin; reverse: dark yellow (Pr. Co-4-b), dark brown in submarginal areas; soluble pigments: doubtful.

III. Melanoid pigments

Peptone-yeast extract agar: negative after 24 hours, positive after 48 hours; tyrosine agar: positive after 24 hours, positive after 48 hours.

IV. Morphology of the sporulating aerial mycelium

Section: spira-retinaculum apertum. Sympodial branched type. Spirals often irregular, with up to 5 coils often of different diameters.

V. Utilization of carbon sources

No growth or only sparing growth on arabinose, xylose, inositol, mannitol, fructose, rhamnose, saccharose, raffinose.

u
P VI. Spores

Oval to cylindrical-oval, sometimes of irregular size, smooth-walled. Spore chains with more than 10 spores.

Cu
P 3
P VII. Nonomura diagram

R(Cy) 100 SRA sm(±) (±) (±) ~~ANALYST~~

P All Streptomycetes strains which produce the lipase inhibitor lipstatin are suitable for the purpose of the present invention, especially Streptomyces toxytricini 85-13, NRRL 15443, and its subcultures, mutants and variants. ✓

10
15
20
25
30
The cultivation of these microorganisms for the manufacture of lipstatin can be carried out according to various fermentation methods. It can be carried out, for example, in shaking flasks or in 10 l or 200 l and 1000 l fermentors. A fixed amount of spore material or mycelium of a lipstatin-producing strain is introduced into a liquid medium which contains suitable carbon and nitrogen sources and salts required for the growth and the mixture is aerobically incubated at a temperature of 20°-37°C for 1-6 days. Suitable carbon sources are, for example, dextrin, glucose, starch, ribose and glycerine. Suitable nitrogen sources are, for example, yeast extract, peptone or soya meal. Preferred salts are ammonium, magnesium and calcium salts. The fermentation is carried out at pH 6-8. W

30
The isolation of the lipstatin is carried out according to methods which are known per se and which are familiar to any person skilled in the art. For example, it can be carried out as follows:

35
After completion of the fermentation the fermentation broth is centrifuged, whereupon 60-80% of the activity is found in the cell mass and the remainder is found in the

centrifugate. The cell mass can then be treated with a lower alcohol such as methanol and ethanol and extracted with the same solvent. The centrifugate can be extracted with a suitable organic solvent (e.g. with methylene chloride or ethyl acetate). The material produced from the extracts contains the desired lipstatin and can be enriched and purified by chromatographic methods. Suitable methods are, for example, multiplicative extraction with the system hexane/methanol/water (50:40:9), filtration chromatography over silica gel while eluting with chloroform, column chromatography on silica gel while eluting with hexane, ethyl acetate and mixtures thereof, chromatography on apolar carrier materials while eluting with polar solvents such as methanol (reversed-phase chromatography) and high pressure liquid chromatography.

The Examples hereinafter contain detail information relating to the cultivation of *Streptomyces toxytricini* 85-13 and the isolation of the lipstatin.

Tetrahydrolipstatin, the compound of formula Ib, can be manufactured by hydrogenating lipstatin in the presence of a suitable catalyst. Examples of catalysts which can be used are palladium/carbon, platinum oxide, palladium and the like. Suitable solvents are, for example, lower alcohols such as methanol and ethanol. The hydrogenation is preferably carried out at low hydrogen pressures and at room temperature (about 23°C.)

The compounds of formula I can be used as medicaments, for example in the form of pharmaceutical preparations. Illustratively, the pharmaceutical preparations can be administered orally, for example in the form of tablets, coated tablets, dragées, hard and soft gelatine capsules, solutions, emulsions or suspensions. If desired, the compounds also can be administered ^aparenterally.

For the manufacture of pharmaceutical preparations the compounds of formula I can be processed with pharmaceutically inert, inorganic or organic carriers. Examples of carriers which can be used for tablets, coated tablets, dragées and hard gelatine capsules are lactose, maize starch or derivatives thereof, talc, stearic acid or its salts and the like. Suitable carriers for soft gelatine capsules are, for example, vegetable oils, waxes, fats, semi-solid and liquid polyols and the like; depending on the nature of the active ingredient no carriers are, however, generally required in the case of soft gelatine capsules. Suitable carriers for the manufacture of solutions and syrups are, for example, water polyols, saccharose, invert sugar, glucose and the like.

Moreover, the pharmaceutical preparations can contain preserving agents, solubilizers, stabilizing agents, wetting agents, emulsifying agents, sweetening agents, colouring agents, flavouring agents, salts for varying the osmotic pressure, buffers, coating agents or antioxidants. They can also contain still other therapeutically valuable substances.

As mentioned earlier, medicaments containing a compound of formula I are also an object of the present invention as is a process for the manufacture of these medicaments, which process comprises bringing a compound of formula I and, if desired, one or more other therapeutically valuable substances into a galenical administration form. As mentioned earlier, the compounds of formula I can be used in the control or prevention of illnesses and especially in the control or prevention of obesity and hyperlipaemia. The dosage can vary within wide limits and is, of course, fitted to the individual requirements in each particular case. In general, in the case of oral administration a daily dosage of about 0.1 mg to 100 mg/kg body weight of compound I is appropriate.

Preferably, the pharmaceutical compositions of formula I in unit dosage form contain about 5 % to 95 % by weight of compound I to the total composition.

5 A range of 10 % to 50 % is preferred.

The compounds of formula I can also be added to industrially-produced foodstuffs, especially to fats, oils, butter, margarine, chocolate and other confectionery
10 goods. Such industrially-produced foodstuffs and their production are also objects of the present invention.

15

20

25

30

35

20 The following Examples illustrate the present invention in more detail, but are not
20 intended to limit its extent. All temperatures are given in degrees Celsius ($^{\circ}\text{C}$). Room
temperature is about 23°C . Unless otherwise indicated, percentages and ratios relating
to solvents are expressed in volume, and the remaining percentages are expressed in
5 weight. Unless indicated otherwise, all Examples were carried out as written. A Lobar
column Lichoprep RP-8, size C, is a lowpressure reverse phase
column, commercially available from Merck Co.

10

DE W^h Example 1

15 (a) Fermentation:

15 A shaking flask containing pre-culture medium 391 is
inoculated with spores of Streptomyces toxytricini 85-13
(or vegetative mycelium thereof) and aerobically incubated
70 as a shaking culture at 28°C for 72 hours. About 2-5 vol.%
of this culture is used to inoculate a fermentor pre-
20 culture of 10 l containing pre-culture medium 391. Incu-
20 bation is carried out at 28° for 3 days with aeration of 1
vvm and stirring at 400 rpm. This 10 l pre-culture is used
to inoculate a 200 l production fermentor containing pro-
70 duction medium N7. Fermentation is carried out at 28° for
25 124 hours with aeration of 1.0 vvm and stirring at 150 rpm.
Regular analyses show after 124 hours an extracellular
lipase-inhibiting activity of 53 IC_{50}/ml .

30

35

The pre-culture medium 391 (pH 7.0) has the following composition: 3% maize starch, 4% dextrin, 3% soya meal, 0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.6% CaCO_3 and 0.8% soya oil. The pH was adjusted to 7. The production medium N 7 (pH 7.0) has the following composition: 1% potato starch, 0.5% glucose, 1% ribose, 0.5% glycerine, 0.2% peptone, 2% soya meal and 0.2% $(\text{NH}_4)_2\text{SO}_4$.

Working-up:

The fermentation broth is centrifuged by means of a tube centrifuge, whereby there are obtained 175 l of culture filtrate and 12 kg of mycelium. The mycelium is discarded. The culture filtrate is heated to 80° for 10 minutes, cooled, again centrifuged and concentrated to 50 l at 30° in vacuo. This concentrate is extracted with 50 l of hexane using a continuously operating extractor, the emulsion obtained is mixed with 50 l of hexane/ethyl acetate (1:1) and the organic phase is separated. This is dried over sodium sulphate and evaporated, there being obtained 199 g of crude extract I. The aqueous phase is diluted with water to 100 l and extracted with 100 l of ethyl acetate. After evaporation of the ethyl acetate solution, there are obtained 49 g of crude extract II. The aqueous phase is subsequently extracted once more with 100 l of ethyl acetate, whereby 78 g of crude extract III are obtained after evaporation.

Purification:

The crude extracts II and III are filtered in three portions over in each case 1 kg of silica gel 60 (0.040-0.063 mm particle size), whereby the elution is carried out with chloroform (column: 10 x 100 cm). 18.3 g of enriched material are obtained in this manner. 178 g of this substance are again filtered over 1 kg of silica gel while

eluting with chloroform. 5.29 g of active material are thus obtained. 802 mg of this substance are purified by reversed-phase chromatography on a commercially obtainable Lobar column (Lichoprep RP-8, size C) while eluting with methanol. There are obtained 158 mg of (2S,3S,5S,7Z,10Z)-5-[(S)-2-formamido-4-methyl-valeryloxy] -2-hexyl-3-hydroxy-7,10-hexadecadienoic acid lactone (lipstatin) which is a yellowish oil at room temperature. It is waxy-crystalline at low temperatures.

10

P 30 Microanalysis (dried for 20 hours in a high vacuum at 50°):

P 2 Calculated for $C_{29}H_{49}N_1O_5$ (491.713): C 70.84, H 10.04,
N 2.85.
Found C 70.85, H 9.97,
N 2.59.

15

P 32 Optical rotation: $[\alpha]_D^{20} = -19.0^\circ$ (c = 1 in chloroform).
31 20 32

P 30 Mass spectrum (chemical ionization with NH_3 as the reagent gas): Peaks at, inter alia, m/z 509 ($M+NH_4^+$) and 492 ($M+H^+$).

P IR spectrum (film): Bands at, inter alia, 3318, 3012, 2928, 2558, 2745, 1823, 1740, 1673, 1521, 1382, 1370, 1250, 1191 cm^{-1} .

31

25

P The absolute configuration could be established by chemically degrading the lipstatin and comparing the fractions obtained with known substances.

30

35

Example 2

Fermentation

A 200 l fermentor containing production medium N 16 is inoculated with a pre-culture of Streptomyces toxytricini 85713 (shaking flasks and then 10 l fermentation) prepared in accordance with Example 1. The production medium N 16 corresponds to production medium N 7 used in Example 1, but also contains 0.1% pig lard. The fermentation is carried out as described in Example 1 for 120 hours. After 120 hours, the intracellular lipase-inhibiting activity amounts to 71 IC₅₀/ml of fermentation broth and the extracellular lipase-inhibiting activity amounts to 4 IC₅₀/ml of fermentation broth.

Working-up

After completion of the fermentation, the fermentation broth is heated to 80° for 10 minutes, subsequently cooled and the cell mass is separated using a tube centrifuge. By two-fold centrifugation there are obtained 11.4 kg of mycelium; the culture filtrate is discarded. The mycelium is triturated in 70 l of methanol for 30 minutes, whereupon the suspension obtained is suction filtered. The filter cake is again triturated with 50 l of methanol and suction filtered. The combined methanolic extracts are concentrated to 1.8 l. This concentrate is extracted three times with 2 l of butyl acetate each time. 160 g of crude extract are obtained from the combined organic phases after evaporation.

u(g) Purification:

This crude extract is purified by multiplicative extraction with the system hexane/methanol/water (5:4:0:9). The active substance is firstly transferred from the lower phase (lp) into the upper phase (up). 160 g of crude extract are dissolved in 4 l of lp and stirred in a stirring vessel with 4 l of up. After separating the up, the lp is extracted a second time with 4 l of fresh up. A stable emulsion forms and to this there are added 4 l of lp and 4 l of up, whereupon a good phase separation is achieved. After separating the up, the lp is extracted twice more with 8 l of fresh up. The combined up give 90.3 g of extract after evaporation. The extracted lp is discarded. The active substance is now transferred from the up into the lp. 90.3 g of the above extract are dissolved in 4 l of up and extracted with 4 l of lp. After phase separation, the up is extracted a further three times with fresh lp. The up is subsequently discarded. The combined lp are concentrated to 0.7 l of aqueous phase and this is extracted eight times with a total of 0.2 l of ethyl acetate. 25.8 g of product are obtained after evaporation. The extracted aqueous phase is discarded. The further purification of this material is carried out by filtration over 1 kg of silica gel 60 (0.040-0.063 mm particle size; column 10 x 100 cm) while eluting with chloroform. There are obtained 649 mg of product which is chromatographed on a Lobar column (Lichoprep RP-8, size C) while eluting with methanol (reversed-phase chromatography). There are obtained 204 mg of lipstatin which is pure according to thin-layer chromatography.

Example 3

138 mg of lipstatin are dissolved in 10 ml of ethanol and the solution is treated with 60 mg of 5 percent palladium/carbon and stirred at room temperature for 3 hours in a hydrogen atmosphere (balloon). The catalyst is subsequently centrifuged off. The hydrogenation product is chromatographed over a short silica gel column (1 x 5 cm) with chloroform. There are obtained 112 mg of (2S,3S,5S)-5-[(S)-2-formamido-4-methyl-valeryloxy]-2-hexyl-3-hydroxy-hexadecanoic acid lactone (tetrahydrolipstatin) as a waxy, slightly yellow solid.

Optical rotation: $[\alpha]_D^{20} = -32.0^\circ$ (c = 1 in chloroform).

Mass spectrum (chemical ionization with NH_3 is the reagent gas): Peaks at, inter alia, m/z 513 ($\text{M}+\text{NH}_4^+$); 496 ($\text{M}+\text{H}^+$) and 452 ($\text{M}+\text{H}^+-\text{CO}_2$).

IR spectrum (film): Bands at, inter alia, 3332, 2956, 2921, 2853, 1838, 1731, 1709, 1680, 1665, 1524, 1383, 1249 and 1200 cm^{-1} .

$^1\text{H-NMR}$ spectrum (270 MHz, CDCl_3): 0.89 (6H); 0.97 (6H); 1.15-1.5 (27H); 1.5-1.85 (6H); 1.9-2.25 (2H); 3.24 (1H); 4.32 (1H); 4.68 (1H); 5.03 (1H); 6.43 (1H); 8.07 and 8.21 (1H) ppm.

Example 4

(a) Fermentation:

A 2 l shaking culture flask containing pre-culture medium 391 is inoculated with spores of an agar slant culture of *Streptomyces toxytricini* 85-13 and aerobically incubated at 28°C for 72 hours. Thereafter, the 2 l pre-culture is transferred into a 50 l fermentor containing production medium N 16 and incubated at 28°C for 77 hours with 0.5 vvm aeration. This 50 l pre-culture is used to

20 inoculate a 1000 l fermentor containing medium N 16. This production fermentation is carried out at 28°C and 0.5 vvm aeration for 91 hours, whereby a lipstatin titre of 73 IC₅₀/ml intracellularly and 16 IC₅₀/ml extracellularly is achieved. The entire fermentation broth is cooled to 2°C and centrifuged, whereby there are obtained 41 kg of moist biomass which are frozen at -20°C.

3120 (b) Working-up

10 37 kg of mycelium are melted at 4°C and homogenized with about 40 l of water in a mixer. The thinly liquid suspension obtained is treated with 140 l of methanol and stirred for 20 minutes. The mixture is subsequently suction filtered over a cloth filter, whereupon the filter cake is extracted further with 140 l of methanol. The 15 methanol extracts are concentrated at 30°C to about 22 l. The concentrate obtained is diluted with water to 50 l and extracted three times in a stirring vessel with 50 l of hexane/ethyl acetate (1:1) each time. In the second and 20 third extractions there are obtained emulsions which can be broken by the addition of about 1.4 kg and 0.5 kg of sodium chloride, respectively. The combined organic extracts are concentrated, dried over sodium sulphate and evaporated to an oily residue. 428 g of crude extract are obtained.

25

(c) Purification

This crude extract is filtered in four portions over in each case 1 kg of silica gel 60 (0.040-0.063 mm particle size), whereby the elution is carried out with chloroform (column: 10 x 100 cm). There are obtained 70 g of enriched preparation which is filtered in two portions over in each case 1 kg of silica gel 60 while eluting with hexane/ethyl acetate (gradient from 9:1 to 4:1). There are obtained 4.2 g of active material which is purified in four portions 35 by reversed-phase chromatography on a Lobar column (Lichoprep RP-8, size C) while eluting with methanol. 1.77 g of lipstatin are obtained.

Example A

Manufacture of soft gelatine capsules of the following composition:

5	<u>10250*</u>	<u>Amount per capsule</u>
	Lipstatin	50 mg
	NEOBEE M-5	450 μ l

10 P The solution of the active substance in NEOBEE M-5 is filled into soft gelatine capsules of suitable size. NEOBEE M-5 is a mixture of triglycerides commonly used for pharmaceutical preparations.

15

20

25

30

35 -